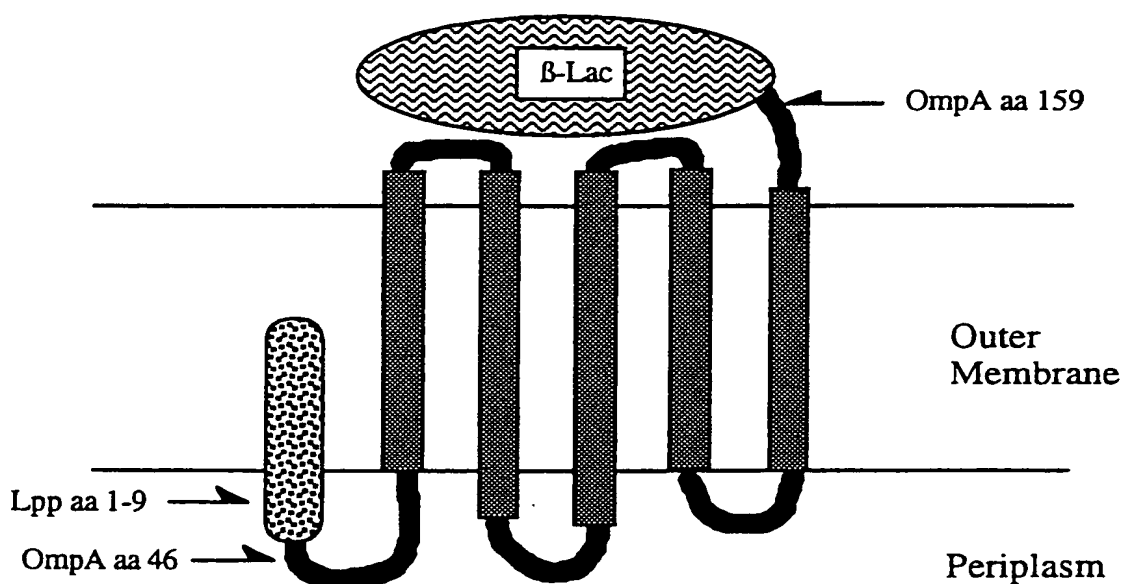


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**(54) Title:** EXPRESSION OF PROTEINS ON BACTERIAL SURFACE**(57) Abstract**

The invention relates to a method for producing stable, surface-expressed polypeptides from recombinant gram-negative bacterial cell hosts. A tripartite chimeric gene and its related recombinant vector include separate DNA sequences for directing or targeting and translocating a desired gene product from a cell periplasm to the external cell surface. A wide range of polypeptides may be efficiently surface expressed, including  $\beta$ -lactamase and alkaline phosphatase. Full enzyme activity is maintained and the proteins remain anchored to the bacterial outer membrane surface.

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DESCRIPTIONEXPRESSION OF PROTEINS ON BACTERIAL SURFACE

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The United States Government may have certain rights in the present invention pursuant to Grant No. BCS-9013007 awarded by the National Science Foundation.

10

BACKGROUND OF THE INVENTIONField of the Invention

15 The invention relates generally to the exportation and localization of polypeptides to the external membrane surface of a gram-negative cell, to recombinant vectors useful for the transformation of a host cell and to chimeric genes that provide outer membrane targeting and transmembrane sequences. Methods are disclosed providing  
20 for surface expression of proteins, including antigenically active proteins, specific binding proteins and enzymatically active species.

Description of Related Art

25

There is substantial interest in the expression of selected proteins on the surface of bacteria. Many potential applications exist, including the production of genetically engineered whole cell adsorbents,  
30 construction of "peptide libraries" where bacteria carry different exposed sequences, cell-bound enzymes (another form of immobilization), and use as live vaccines or immunogens to generate antibodies.

35

One approach to obtaining surface expressed foreign proteins has been to use a native membrane protein as a carrier for a foreign protein. LamB, an outer membrane

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protein of *Escherichia coli*, has been fused with peptides varying in length up to about 60 amino acids with successful expression of the hybrid protein at a recombinant host cell surface (Charbit, et al., 1991).

5 Unfortunately, only relatively short polypeptides are surface-expressed using this method. Outer membrane proteins have "loop" regions spanning the membrane surface and while it is possible to substitute foreign DNA into the gene regions encoding the loop regions,  
10 there are only a limited number of insertions possible, constrained by the size of the loop region and, apparently, by the requirement to preserve the penetration and translocating properties of the membrane protein.

15

In general, attempts to develop methods of anchoring larger proteins as well as the smaller peptides on a bacterial cell surface have focused on fusion of the desired recombinant polypeptide to a native protein that  
20 is normally exposed on the cell's exterior in hope that the resulting hybrid will also be localized on the surface. The problem with this approach is that fusion of the foreign protein interferes with localization and, in many cases, the hybrid molecule is unable to reach the  
25 cell surface.

Nevertheless, in one example employing the *Klebsiella* enzyme pullulanase, a normally periplasmic protein,  $\beta$ -lactamase, was translocated through the *E. coli* outer membrane. C-terminal regions of pullulanase  
30 were replaced with DNA segments encoding  $\beta$ -lactamase or alkaline phosphatase. Only the hybrid protein with  $\beta$ -lactamase was transported to the cell surface (Kornacker and Pugsley, 1990). However, the surface-expressed  
35 protein was only transiently anchored to the cell surface, suggesting a severe limitation on the potential value of any other proteins expressed by this method as

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surface immunogens, adsorbents, or surface immobilized species. Furthermore, the assembly of pullulanase fusions into the cell surface is a very complicated process requiring the presence of at least 14 foreign gene products in the host cell. It should be noted that alkaline phosphatase fused to the same pullulanase sequence could not be localized on the cell surface (Kornacker and Pugsley, 1990).

10       The mechanisms of protein insertion within- and translocation across- the outer membrane of gram-negative bacteria are not well understood. For some outer membrane proteins, such as the PhoE porin, the information necessary for proper localization and  
15 assembly is interspersed within the primary sequence (Bosch et al., 1986; Bosch et al., 1989). Alternatively, the targeting signal may be contained within a single short continuous segment. For example, the first nine N-terminal amino acids of the major *E. coli* lipoprotein are  
20 necessary for proper localization in the outer membrane. Fusion to this short sequence is sufficient to direct the normally soluble periplasmic protein  $\beta$ -lactamase to the outer membrane (Ghrayeb and Inouye, 1984). Similarly, extensive studies with OmpA have suggested that the  
25 region between residues 154 and 180 is crucial for localization (Klose et al., 1988a, 1988b). With OmpA, targeting and outer membrane assembly appear to be distinct events. Only large fragments containing the entire membrane spanning sequence of OmpA are able to  
30 assemble into a conformation exhibiting native resistance to proteolytic digestion (Klose et al., 1988a).

35       In general, amino acid substitutions or insertions within outer membrane loops exposed on the cell surface are well tolerated and do not interfere with the folding of the protein in the membrane. Peptides as large as 60 amino acids have been inserted within external loops of

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various outer membrane proteins and appear to be exposed on the surface of intact *E. coli* cells as indicated by immunochemical techniques (Charbit et al., 1991). However, efforts to direct soluble reporter proteins such as alkaline phosphatase, to the cell surface using outer membrane protein fragments have not been successful. These fusions either end up at incorrect cellular locations or become anchored in the membrane with the secreted protein domain facing the periplasm (Murphy et al., 1990). In gram-negative bacteria the outer membrane acts as a barrier to restrict the export of proteins from the cell. Normally only pilins, flagellins, specific enzymes and a few toxins are completely transported across the outer membrane (Kornacker and Pugsley, 1990). Most of these proteins are first secreted into the periplasmic space via the general secretion pathway and then cross the outer membrane by a process that involves the action of several additional gene products (Filloux et al., 1990).

Whole cell adsorbents are considered to have potential value for biotechnology applications for the purification of various molecules or the selective removal of hazardous compounds from contaminated waste waters. However, a major constraint in the development of whole cell adsorbents is the availability of bacterial strains with suitable ligands on their surface. Although functional antibody fragments have been produced in *Escherichia coli* (Skerra and Pluckthun 1988, Better et al. 1988, Orlandi et al. 1989, Sastry et al. 1989), these polypeptides have not been expressed on the cell surface. Indeed, a "library" of recombinant immunoglobulins containing both heavy and light variable domains (Huse et al. 1989) has been produced with the proteins having antigen-binding affinity comparable to the corresponding natural antibodies. Furthermore, the variety of recombinant immunoglobulins from bacteria is greater than

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the number of antibody molecules that can be generated by the mammalian cell. In this way it has become possible to expand the repertoire of antibodies that can be made by the immune system (Hus t al. 1989). While the  
5 availability of such a wide range of immunoglobulins suggests the potential for creation of *E. coli* cells endowed with immunological surface receptors, there has been little success in producing recombinant proteins on the surface of bacterial cells, and conspicuous lack of a  
10 method to generate recombinant immunoglobulins on surfaces of gram negative host cells.

Although the potential repertoire of immunoglobulins produced in an immunized animal is high ( $>10^{10}$ ), only a  
15 small number of monoclonal antibodies can be generated using hybridomas. This limitation complicates the isolation of antibodies with specific properties, such as the ability to act as a catalyst. Combinational antibody libraries comprising millions of genes of different  
20 antibodies have been cloned using phage  $\lambda$  (Huse et al., 1989). However, screening the library to select the desired clone can be extremely time consuming and complicated. One approach to the screening problem has been an attempt to express antibodies on the surface of  
25 filamentous phage. Phage particles displaying high affinity antibody molecules on their surface can be enriched by chromatography through a column of immobilized antigen (Barbas et al., 1991; Clarckson et al., 1991; Breitling, 1991). Although the feasibility of  
30 this technique has been demonstrated, several problems are apparent, including: (1) fusion to bacteriophage coat proteins causing interference with antibody folding, (2) subcloning of large numbers of positive phage particles in order to produce soluble antibody fragments to carry  
35 ut more xtensiv characterization, and (3) lack of control of the number of antibody molecules on the phage

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surface, thus affecting binding to the immobilized antigen and complicating the selection procedure.

#### SUMMARY OF THE INVENTION

5

The present invention addresses one or more of the foregoing problems in providing a versatile recombinant vector that will promote transport of a periplasmic or other protein to the external face of the outer membrane of a gram-negative bacterial cell in the absence of any specific export components. In particular, the vector includes a tripartite chimeric gene having a membrane targeting sequence, a membrane translocating sequence capable of locating a fusion protein on the outer surface and a gene segment encoding any of a variety of proteins.

Overall and in general the tripartite chimeric genes of the invention include at least three DNA segments. One segment is a targeting DNA sequence encoding a polypeptide capable of targeting and anchoring the fusion polypeptide to a host cell outer membrane. Targeting sequences are well known and have been identified in several of membrane proteins including Lpp. Generally, as in the case of Lpp, the protein domains serving as localization signals are relatively short. The Lpp targeting sequence includes the signal sequence and the first 9 amino acids of the mature protein. These amino acids are found at the amino terminus of Lpp. *E. coli* outer membrane lipoproteins from which targeting sequences may be derived include TraT, OsmB, NlpB and BlaZ. Lipoprotein 1 from *Pseudomonas aeruginosa* or the PA1 and PCN proteins from *Haemophilus influenza* as well as the 17 kDa lipoprotein from *Rickettsia rickettsii* and the H.8 protein from *Neisseria gonorrhoea* and the like may be used.



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A second component of tripartite chimeric genes is a DNA segment encoding a membrane-transversing amino acid sequence. Transversing is intended to denote an amino acid sequence capable of transporting a heterologous or homologous polypeptide through the outer membrane. In preferred embodiments, the membrane transversing sequence will direct the fusion polypeptide to the external surface. As with targeting DNA segments, transmembrane segments are typically found in outer membrane proteins of all species of gram-negative bacteria. Transmembrane proteins, however, serve a different function from that of targeting sequences and generally include amino acid sequences longer than the polypeptide sequences effective in targeting proteins to the bacterial outer membrane. For example, amino acids 46-159 of the *E. coli* outer membrane protein OmpA effectively localize a fused polypeptide to the external surface of the outer membrane when also fused to a membrane targeting sequence. These surface exposed polypeptides are not limited to relatively short amino acid sequences as when they are incorporated into the loop regions of a complete transmembrane lipoprotein. While the invention has been demonstrated with a transmembrane directing protein sequence from OmpA, other transmembrane directing sequences from outer membrane proteins may be employed.

The third gene segment comprising the tripartite chimeric gene fusion is a DNA segment that encodes any one of a variety of desired polypeptides. This DNA segment is positioned downstream from the DNA segment encoding the transmembrane sequence. The tripartite chimeric gene when provided with a functional promoter is expressible in gram-negative host cells.

A particular embodiment of the invention includes recombinant vectors prepared from the herein-described tripartite chimeric gene fusions. Such vectors will

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express fusion polypeptides at the outer membrane cell surface of a gram-negative host cell. These recombinant vectors include a functional promoter sequence and a targeting DNA sequence encoding a protein capable of targeting to the outer surface of a gram-negative bacterial host cell. The targeting gene is typically positioned downstream of the promoter sequence. A transmembrane gene sequence is positioned downstream of the targeting gene sequence. The transmembrane sequence will encode a protein domain capable of transversing the cell outer membrane. The vector will also include a DNA sequence which encodes a desired protein. This sequence when positioned downstream of the transmembrane sequence will be expressed on the external surface of the outer membrane, and typically is exposed to the external medium while remaining stably anchored to the membrane surface.

A most preferred embodiment of the recombinant vector is plasmid pTX101. This plasmid contains a fusion of the signal sequence and the first 9 amino acids of the major outer membrane lipoprotein of *E. coli*, a 342-base pair fragment from the outer membrane protein OmpA and the coding sequence for the complete mature  $\beta$ -lactamase protein. However, clearly numerous variations of the disclosed recombinant vectors could be prepared using techniques well known to those of skill in the art. DNA sequences encoding regions from a wide variety of membrane proteins could be employed. Such regions may be fused with any of a number of genes or gene fragments via a polylinker region.

The polypeptides encoded by the nucleic acid segments identified herein have been described in terms of function related to targeting and transversing fusion polypeptides to a gram-negative bacterial cell outer membrane surface. The invention is intended to include variations of the fused genes disclosed to the extent

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that the encoded polypeptides are functionally biologically equivalent. In general, by biologically functionally equivalent is meant amino acid sequences that may vary from certain of the disclosed fusion products, by e.g., natural genetic drift, strain or subspecies antigenic variation or by mutation of the DNA molecules without loss of appropriate membrane targeting or transverse functions as described.

10 Likewise, certain changes in nucleic acid composition of genes encoding polypeptides having the aforementioned functions, will not affect the general broad concept of the invention. For example, vectors containing variant codons for a particular amino acid, 15 while altering the DNA composition, will not change the amino acid identity. Minor base pair changes, while producing some variation in primary amino acid sequence of the encoded polypeptide, are not expected to substantially alter function. All such variations, 20 whether in amino acid or nucleic acid composition, are contemplated to be within the scope of the invention.

The methods illustrated for the expression of desired recombinant polypeptides on the cell surface may 25 also be achieved by fusion to protein domains other than those derived from the major lipoprotein and OmpA, provided that these domains can function for the expression of the desired polypeptide on the cell surface. Generally, the desired polypeptide is fused to 30 an amino acid sequence that includes the signals for localization to the outer membrane and for translocation across the outer membrane. The amino acid sequences responsible for localization and for translocation across the outer membrane may be derived either from the same 35 bacterial protein or from different proteins of the same or different bacterial species. Examples of proteins that may serve as sources of localization signal domains

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include *E. coli* outer membrane lipoproteins from such as *TraT*, *OsmB*, *NlpB*, *BlaZ*, *Pseudomonas aeruginosa* lipoprotein 1, *Haemophilus influenza* PA1 and PCN proteins, *Rickettsia rickettsii* 17 kDa lipoprotein, *Neisseria gonorrhoea* H.8 protein and the like. A sequence that spans the outer membrane and serves to transport the desired recombinant polypeptide to the cell surface can be derived from a membrane spanning domain of suitable length from any native outer membrane protein of gram-negative bacteria, including the porins *LamB*, *PhoE*, *OmpC* and *OmpF*, as well as other outer membrane proteins such as *OmpT*, *FepA*, and the like.

Any of a wide variety of gram-negative bacteria may be useful in practicing the invention. Such gram-negative bacteria include *E. coli*, *Salmonella*, *Klebsiella*, *Erwinia*, and the like. *E. coli* and *Salmonella* are particularly preferred as host cells. Although there are variations among the bacteria outer membrane proteins are similar. Target and transversing sequences from any of the membrane proteins may be used in constructing vectors useful for exportation across the cell wall of gram-negative bacteria.

Another aspect of the invention includes transformants. A typical transformant is a *Salmonella* prepared by transformation with the described recombinant vectors. A most preferred transformant is *E. coli*.

The invention is typically practiced using one or more of the commonly available gram-negative bacteria as cell hosts. However, rough mutants having somewhat differing membrane compositions are expected to also be useful in the practice of the invention. Membranes with higher phospholipid content, for example, may for some fusion polypeptides, provide more efficient surface expression at higher temperatures. Alternatively, it may

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be desirable to anchor some polypeptides closer to the membrane surface with increased lipid-protein interactions, perhaps for the purpose of increasing immunogenic response or altering adsorbent properties.

- 5 Such mutants, spontaneously generated or otherwise, are contemplated as useful as host cells and/or as sources for membrane directing and transversing sequences.

10 Numerous types of fusion polypeptides may be expressed using the aforementioned system. Relatively large proteins such as alkaline phosphatase have been expressed on the surface of *E. coli* host cells. In its dimeric form alkaline phosphatase has a molecular weight of greater than 80 kDa. Other large proteins are also  
15 expected to be effectively surface expressed. Examples of expressed polypeptides include  $\beta$ -lactamase, alkaline phosphatase, cellulose binding domain of cellulase, or single-chain F<sub>v</sub> antibody.

20 Expression of a variety of single-chain antibodies on the surface of a gram-negative bacterial host cell has several potential important applications particularly for the preparation of whole cell adsorbents. In addition, a variety of antigenic determinants may be expressed on a  
25 cell surface and used to prepare bacterial vaccines. A selected antigen in combination with an activating agent such as IL-4 on the surface of a bacterium may have potential use in stimulating an immune response toward a surface exposed antigen.

30

Tripartite chimeric gene fusions or the recombinant vectors herein described will typically include appropriate promoters. Such promoters are well known to those of skill in the art and examples include *lpp*  
35 promoter or *lac* promoter. Additionally, recombinant vectors also include a signal peptide. In preferred

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embodiments the signal peptide is positioned upstream of the targeting gene segment in recombinant vectors.

5 The invention also includes a method for expressing a fusion polypeptide anchored on the outer membrane surface of a gram-negative bacterial host cell. A gene segment encoding a desired polypeptide is selected and inserted by the herein described methods into one or more of the disclosed recombinant vectors. A selected gram-negative cell is transformed with the vector. The transformants are cultured and screened in order to identify clone transformants having a desired peptide expressed on the host cell surface. There are numerous ways the desired gene segment encoding the polypeptide could be incorporated into one or more of the disclosed recombinant vectors. For example, plasmid pTX101 may be cut with the restriction endonuclease *EcoRI* at the unique site in the linker region between the *OmpA* and  $\beta$ -lactamase sequence. Typically, blunt ends are created on the DNA by treating with the Klenow fragment of DNA polymerase. Plasmids containing the coding sequence for the desired polypeptide may be isolated and DNA fragments obtained by cutting that plasmid with an appropriate endonuclease followed by blunt ending again using a Klenow fragment or similar polymerase. The linearized pTX101 vector and the desired gene fragment may then be ligated and the resultant DNA transformed into an appropriate bacterial host cell strain such as *E. coli* strain JM109.

30

Surprisingly, the temperature at which the cells are cultured has an effect on the expression of the desired polypeptide. Culturing at higher temperatures, about 40°C, for example, results in less efficient expression of the desired polypeptide on the surface of the bacterial host. Although expression on the surface may be obtained when culturing is performed between about

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22°-40°C, a preferred temperature range is between 22°-27°C and a most preferred temperature being around 24°C.

Yet another aspect of the present invention is a method for obtaining an immunogenic polypeptide. An immunogenic polypeptide to which it is desired to elicit an immune response is selected and then inserted into an appropriate recombinant vector prepared in accordance with the aforementioned procedures. Appropriate gram-negative cells are transformed and the culture screened for transformants. Transformants are then screened to determine the degree of immunogenicity and those that are highly immunogenic are used to obtain one or more antibodies. This method is particularly useful because it is known that surface expressed polypeptides typically elicit higher antigenic and immunogenic responses than those peptides that are not immobilized on a bacterial surface. Surface exposed immunogenic polypeptides may also be used to prepare vaccines, typically by mixing the cells in a pharmaceutically acceptable vehicle suitable for administration in mammals.

Antibodies can be equally well expressed on the surface of the cell. When such antibodies are expressed on cell surfaces those with high affinity for particular antigens may be selected. Variants of antibodies may be prepared and surface expressed and antibody-like sequences may be prepared and tested for affinity to the appropriate antigens.

30

In yet another aspect of the invention it is contemplated that kits useful for transforming gram-negative bacterial host cells may be prepared. Kits will include at least one recombinant vector prepared in accordance with the herein described invention in an appropriately compartmentalized container. A preferred recombinant vector is defined by SEQ ID NO:1.

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The invention also includes a method for removing contaminants from fluids. In this method various receptor proteins expressed on the outer membranes of gram-negative bacteria may be used to selectively interact with a wide variety of undesirable compounds. Metallothionein, for example, binds with a wide variety of heavy metals including iron, cadmium, zinc, copper, vanadium, and similar metals. When bound to the surface of a gram-negative organism this protein is expected to efficiently remove heavy metals from aqueous samples.

Whole cell adsorbents, with surface expressed polypeptides, such as selected antibodies, may be used to remove biological contaminants, for example, bacterial endotoxin from water samples. The efficiency of such whole cell adsorbents may be increased by cross-linking the bacterial surface. This also may increase the stabilization of the cells against disruption. One method of stabilization involves the specific cross-linking of the cells through the lipopolysaccharide component of the surface. Thus the cells can be aggregated and stabilized without affecting the function of surface-expressed proteins. Other types of cell adsorbents are contemplated including the use of cellulose binding domains, starch binding domains, protein A, lectins, or protease receptors expressed on outer membrane bacterial cell surfaces.

Still further embodiments of the invention include immobilized enzyme systems. Any one of a wide variety of biocatalytically active polypeptides may be expressed on the surface of a bacterial cell using the disclosed methods. Advantages of having an enzyme expressed on the bacterial cell surface include increased accessibility to substrates, stability, and potentially increased lipid solubility. In a more particular embodiment, biocatalytically active polypeptides immobilized on host



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cell membranes without additional bacterial host cell components may be used in biphasic reaction systems. Enhanced lipid solubility of the immobilized enzymes enable catalyst substrate interaction in the lipophilic solvents with extraction of the water soluble products into the aqueous phase. Further contemplated embodiments in such an immobilized system include encapsulating immobilized enzymes on membrane surfaces within liposomes or similar vesicles.

10

As part of the invention, kits useful for the expression of fusion proteins are also envisioned comprising a container having suitably aliquoted reagents for performing the foregoing methods. For example, the containers may include one or more vectors, examples being the vectors of claim 2, particular embodiments of which are shown schematically in Figure 5. Suitable containers might be vials made of plastic or glass, various tubes such as test tubes, metal cylinders, ceramic cups or the like. Containers may be prepared with a wide range of suitable aliquots, depending on applications and on the scale of the preparation. Generally this will be an amount that is conveniently handled so as to minimize handling and subsequent volumetric manipulations. Most practitioners will prefer to select suitable nucleases such as *EcoRI*, *BamHI*, or *PstI* from common supplies usually on hand; however, such restriction endonucleases could also be optionally included in a kit preparation.

30

Vectors supplied in kit form are preferably supplied in lyophilized form, although such DNA fragments may also be taken up in a suitable solvent such as ethanol, glycols or the like and supplied as suspensions. For most applications, it would be desirable to remove the solvent which for ethanol, for example, is a relatively simple matter of evaporation.

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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic diagram of the Lpp-OmpA- $\beta$ -lactamase fusion in the outer membrane of a gram negative bacterium. Rectangles represent membrane-spanning  $\beta$ -strands of OmpA.

Figure 2 shows the fractionation of membranes from JM109(pTX101) and JM109 cells. The percent of total membrane protein and  $\beta$ -lactamase activity in different fractions from a sucrose gradient of pTX101 is shown.  $\beta$ -lactamase activity was determined from the rate of hydrolysis of penicillin G. Fractions 2-7 had an average density of 1.22 g/cc, 11-13:  $\rho$ =1.19 g/cc and 16-20:  $\rho$ =1.15g/cc corresponding exactly to the values for outer membrane, intermediate and inner membrane vesicles determined by Osborn.

Figure 3 shows fractionation on a sucrose gradient of membranes from JM109(pTX101) and JM109(pJG311). Figure 3A: Samples from every three fractions were pooled together and loaded in consecutive lanes. Lanes 2-8, pTX109: lanes 9-15, pJG311. Lanes: 1: Molecular weight markers: 2: fractions 1-3; 3: fractions 4-6; 4: fractions 7-9; 5: fractions 10-12; 6: fractions 13-15; 7: fractions 16-18; 8: fractions 19-21; 9: fractions 1-3; 10: fractions 4-6; 11: fractions 7-9; 12: fractions 10-12; 13: fractions 13-15; 14: fractions 16-18; 15: fractions 19-21; 16: molecular weight markers. The molecular weight standards (BRL) are: myosin H-chain, 200 kDa: phosphorylase B, 97 kDa: bovine serum albumin, 68 kDa: ovalbumin, 43 kDa: and carbonic anhydrase, 29 kDa. Arrows indicate the fusion proteins Lpp-OmpA- $\beta$ -lactamas (lane 2) and Lpp- $\beta$ -lactamase (lane 9). Figure 3B: Western blot of the JM109(pTX101) fractions from the sucrose gradient (Figure 3A, lanes 2-8). The primary antibody was used at a concentration of 1:20,000. The

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gel was overloaded to show the presence of degradation products. There were no degradation products below the 32.000 dalton molecular weight standard. As with the native  $\beta$ -lactamase, the Lpp-OmpA- $\beta$ -lactamase migrates as two bands depending on the oxidation of the single disulfide bond (30). The prestained molecular weight markers (Bio-Rad) have apparent molecular weights of: 106 kDa, phosphorylase B: 80 kDa, bovine serum albumin: 49 kDa, ovalbumin: 32 kDa, bovine carbonic anhydrase.

10

Figure 4 shows micrographs of the same field of JM109(pTX101) cells labelled with rabbit  $\beta$ -lactamase specific antibodies and rhodamine conjugated rabbit-specific antibodies viewed by fluorescence (4A) and phase contrast microscopy (4B).

15

Figure 5 shows the effect of extended incubation on measured  $\beta$ -lactamase activity of the tripartite fusion expressed on the outer membrane surface of *E. coli*.

20

Figure 6 shows a diagram of the plasmid pTX101.

Figure 7 (SEQ ID NO:1) shows the DNA sequence of the genes coding for the tripartite fusion from pTX101.

25

Figure 8 shows the condons for the segments included in the tripartite fusion from pTX101.

Figure 9 shows a scanning electron micrograph of JM109(pTX101) cells labelled with anti- $\beta$ -lactamase antibodies and secondary gold conjugated antibodies.

30

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTSMaterials and Methods5 Bacterial Strains

*E. coli* strain JM109 (*endA1 recA1 gyrA thi-1 hsdR17(r<sub>k</sub><sup>-</sup>, m<sub>k</sub><sup>+</sup>) relA1 supE44 X(lac-proAB)/F' traD36 proAB lacI<sup>q</sup> lacZAM15*) (Invitrogen, San Diego, CA), ATCC 53323.

10

*Escherichia coli* strain JCB572 is obtained from Dr. J. Beckwith, Department of Molecular Biology and Molecular Genetics, Harvard Medical School, Boston, MA 02115 (Bardwell et al., 1991).

15

Plasmids

Plasmid pSWFII is prepared as described by Ehrmann et al., 1990.

20

Plasmid pJG311 (Ghrayeb and Inouye, 1984; Yamaguchi et al., 1988) was constructed by cutting pMH014 Cm<sup>r</sup> (Yamaguchi et al., 1988) which contains the gene coding for the signal sequence and mature major outer membrane lipoprotein, with *EcoRI* and then removing all the lipoprotein gene except the signal sequence and the first nine amino acids. The  $\beta$ -lactamase gene, cut from pTG206, was ligated into this site creating pJG202. The region coding for the lipoprotein signal sequence, the first nine amino acids from the lipoprotein, and the entire mature  $\beta$ -lactamase, was transferred from pJG202 to the expression plasmid pIN-III-A-Cm<sup>r</sup> (Yoshihiro et al., 1983) to create pJG311, which contains a Lpp- $\beta$ -lactamase fusion. Plasmid pJG311 may also be obtained from Masayori Inouye, Department of Biochemistry, Robert Wood Johnson Medical at Rutgers University of Medicine and

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Dentistry of N w Jersey, Piscataway, New Jersey USA  
08854.

Plasmid pRD87 is constructed in the same manner as  
5 pTU500 (Freudl et al., 1985). pTU500 is constructed by  
cutting the *OmpA* gene from pTU500/1 and ligating it into  
pUC9 (Vieira and Messing, 1982) thereby placing it under  
the control of the *lac* promoter. pRD87 was made  
identically except that the *ompA* gene from pTU500/1 was  
10 cloned into pUC8 (Vieira and Messing, 1982). The two  
plasmids, pTU500 and pRD87, are identical except that  
pTU500 contains an amber mutation at the seventh codon in  
the *ompA* sequence, while pRD87 does not contain the amber  
mutation. Plasmid pRD87 may also be obtained from Ulf  
15 Henning, Max-Planck-Institut für Biologie, Corrensstrasse  
38, D-7400 Tübingen, Germany.

### Cultures

20 Cultures were grown in either LB medium (Difco)  
supplemented with 0.2% glucose or M9 medium supplemented  
with 0.2% casein amino acid hydrolysate and 0.2% glucose.  
The desired antibiotics were added as required.

### 25 General Procedures

SDS-PAGE was performed on 11% and 15% acrylamide  
gels. Protein samples, denatured for 5 minutes in boiling  
SDS containing  $\beta$ -mercaptoethanol, were loaded onto  
30 polyacrylamide gels and run at a constant current. The  
gels were stained with Coomassie brilliant blue (R 250)  
for 15 minutes and the background stain was removed  
overnight with a methanol/acetic acid destaining  
solution.

35

Western blots were performed by running 0.5  $\mu$ g  
protein samples on polyacrylamide gels at constant

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current and were transferred overnight to nitrocellulose membranes. The membranes were incubated for 1 hour with rabbit anti- $\beta$ -lactamase antibodies, rinsed, and incubated for 1 hour with horseradish peroxidase conjugated goat anti-rabbit antibodies. After further rinsing, the membranes were developed with 4-chloro-1-naphthol, which gives a distinct blue color at the sites containing horseradish peroxidase.

10 The enzymatic activity of  $\beta$ -lactamase was measured by the rate of hydrolysis of penicillin G or nitrocefin (Samuni, 1975; O'Callaghan et al., 1972). Hydrolysis of penicillin G gives linear decrease in the adsorption of light at 240 nm, while nitrocefin hydrolysis shows an adsorption increase at 482 nm. The changes in adsorption with time were measured in an LKB spectrophotometer. Protein concentrations were measured by the Bio-Rad assay using standard curves prepared from protein standards and comparing color developed with the reagent measured at 20 595 nm in a spectrophotometer.

The present invention relates to a novel chimeric gene from which a wide variety of recombinant expression vectors useful for surface expression of desired proteins is possible. Appropriately transformed gram-negative host cells will efficiently express proteins on the outer membrane surface without loss of inherent activity. A novel aspect of the fused gene is the use of three separate DNA segments which act respectively (1) to target a fusion product to the host cell outer membrane and (2) to transverse the fusion product across the membrane to the outer surface where (3) the polypeptide expressed by a fused gene of interest becomes stably anchored to the surface.

35

The particular examples herein illustrated utilize recombinant vectors constructed from known DNA segments

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having particular functions. For example, various membrane proteins such as OmpA are known to contain both membran targeting and transversing sequences. However, fusion of alkaline phosphatase with outer membrane proteins has not produced surface expressed alkaline phosphatase (Murphy et al., 1990). The present invention utilizes separate targeting and transversing domains. When engineered into a vector such that a gene for a desired polypeptide product is positioned downstream of both the targeting and transversing sequences, efficient surface expression of the product is effected. Moreover, the targeting sequence is positioned upstream of the transversing sequence.

It will be appreciated that the particular gene sequences shown here to construct a tripartite chimeric gene are not limited to deriving targeting and transversing sequences from lipoprotein and OmpA respectively. Other sequences with analogous function may be used. In particular, this invention may be efficiently practiced with the construct shown schematically in Figure 6 and in particular detail in Figure 7 (SEQ ID NO:1) illustrates useful targeting and transversing DNA sequences fused with the  $\beta$ -lactamase gene, although it is appreciated that numerous other polypeptide sequences could be used rather than  $\beta$ -lactamase.

The invention has numerous applications, a brief background for which is described.

#### Whole Cell Affinity Adsorbents

Affinity purifications of biomolecules rely primarily on the strong interactions between proteins and ligands. Typically, the ligand is bound to a solid support matrix which is employed in a chromatographic-

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type separation. More recently, suspensions of starch granules (Mattiasson and Ling 1986) or liposomes (Powell et al. 1989) have been used as supports for affinity purifications. In some of the most useful and specific separations, the affinity ligands are proteins such as antibodies, lectins or protein receptors (Mohr and Pommerening 1986, Turkova 1978). The preparation of protein affinity adsorbents involves the production, purification and the immobilization of the polypeptide on a solid support matrix. These three steps are generally complicated and often prohibitively expensive for large scale applications. On the other hand bacterial cells expressing proteins on their surface can serve as an important source of low cost solid phase adsorbents.

The human metallothionein gene protein has been expressed as a fusion with an outer membrane protein (Jacobs et al., 1989). Because of the way the fusion protein was constructed, metallothionein was localized on the internal side of the *E. coli* outer membrane, i.e., facing the periplasmic space. Nevertheless, since metal ions can diffuse through the outer membrane, the recombinant cells were able to bind as much as 66 fold more  $Cd^{+2}$  than normal *E. coli*. Another example of a high affinity cellular adsorbents (e.g., Kronvall et al. 1979) includes the use of cultured mammalian cells to remove viral impurities from blood samples (Tsao et al., 1988).

#### Whole Cells As Enzyme Carriers For Bioprocessing

The use of whole cells as enzymatic catalysts has been in use for several years. Typically, a microorganism which produces a certain enzyme is used as a biocatalyst, thus avoiding the costs associated with protein purification and immobilization steps. Usually the cells are first killed, treated with a permeabilizing agent to allow the diffusion of reactants and products



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35

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into the cytoplasm and finally they are stabilized using some form of chemical crosslinking (Tampion and Tampion 1987). Several improvements on the preparation of whole cell biocatalyst have been made over the years. However, certain inherent limitations can not be overcome with the currently available technology. These are: i) The chemical methods which are used for permeabilization of the cell membrane can also result in deactivation of the important enzyme; ii) Other intracellular enzymes may compete for the reaction substrate giving rise to undesired byproducts and decreased yields; and iii) Intracellular degradation processes can limit the functional life of the biocatalyst. Clearly, all these problems can be eliminated if the enzyme is attached to the cell's exterior.

#### Live Bacterial Vaccines

Genetically weakened (attenuated) strains of bacteria that are able to survive and persist in the human or animal body can confer prolonged immunological protection against disease (Stover 1991). Non-recombinant live vaccines have been used for many years for large scale vaccinations (Dougan 1989). For example, live attenuated cultures of *Bacillus Calmette-Guérin* (BCG) which confer immunity against tuberculosis represent the most widely used vaccine in the world (Stover et al. 1991). Recently, emphasis has been shifted to the development of recombinant bacterial vaccines (Curtiss et al. 1989, Charles and Dougan 1990). In this case vaccination consists of the oral administration of a live culture of an attenuated enteric bacterium host such as *E. coli* or *Salmonella typhimurium* which expresses an antigenic peptide from a pathogen. Within the body, some of the bacteria find their way to the intestinal tract where they coexist with the wild type *E. coli* and other enteric microorganisms. In this

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way they ensure the presence of a low level of antigenic peptide in the body. Live vaccines provide more efficient immunity and longer protection against infections compared to subunit or killed bacterial vaccines. There are several reasons for the higher efficacy of live bacterial vaccines (Dougan et al. 1989):

- i) Protection correlates with how long the vaccine is present in the body (De Libero and Kaufman, 1986). Since the bacteria persist in the intestine for very long times, they are able to confer extended immunity; ii) Unlike most currently used vaccines, bacterial vaccines may be administered orally; and iii) Several antigens may be expressed simultaneously in bacteria thus giving rise to multipurpose vaccines.

Although the foregoing antigen may stimulate an immune response even when produced within the cell, the immunogenicity of peptide antigens can be greatly enhanced if they are expressed on the surface of an appropriate host strain (Taylor et al. 1990). This is because the surface of the bacteria such as *Salmonella* or *E. coli* acts as an adjuvant to enhance the immune response to the antigen. The most straightforward way to accomplish this is to insert the foreign peptide within a surface exposed loop of an outer membrane protein which serves as the targeting signal. A fusion protein with the structure outer membrane protein--peptide--outer membrane protein is constructed and then the normal protein localization mechanism of the cell is exploited to carry the peptide to the surface. There appears, however to be an upper limit on the length of foreign polypeptides that can be inserted within outer membrane proteins. The maximum size of foreign sequence that can be accommodated within outer membrane proteins is around 45 to 50 amino acids (Newton et al. 1989, Charbit et al. 1988).

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Several different outer membrane proteins have been exploited as targeting vehicles for the localization of foreign peptides (e.g., Charbit et al., 1988). A number of short amino acid sequences have been inserted within a surface exposed loop of the *E. coli* outer membrane protein maltoporin (LamB) (Charbit et al., 1988). The peptides were localized correctly so that the whole cells could be used to induce an immune response. Expression systems for the localization of antigenic peptides on bacterial surfaces have also been constructed using the *E. coli* K88ac and K88ad pilin proteins (Thiry et al. 1989), the *S. typhimurium* flagellin, (Newton et al. 1989) the TraT lipoprotein (Taylor et al. 1990) and the *E. coli* outer membrane porins PhoE, OmpA and OmpC (Agterberg 1987, Freundl 1989).

Prototype live bacterial vaccines have been prepared using cells having sequences from the influenza virus, cholera toxin B subunit and the gp 120 glycoprotein of HIV-1 expressed on their surface. However, the presence of a fragment of a protein from an infectious agent often does not give satisfactory protection against disease (Dogan et al. 1989).

An advantage of the present invention is the potential to express complete proteins from infectious agents on the surface of the carrier cells. Immunization with an intact protein is more likely to elicit a humoral immune response and provide protective immunity.

30

#### Vaccine Preparation and Use

Preparation of vaccines which contain peptide sequences as active ingredients is generally well understood in the art, as exemplified by U.S. Patents 4,608,251; 4,601,903; 4,599,231; 4,599,230; 4,596,792; and 4,578,770, all incorporated herein by reference.

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Typically, such vaccines are prepared as injectables either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The active immunogenic ingredient is often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like, and combinations thereof. In addition, if desired, the vaccine may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, or adjuvants which enhance the effectiveness of the vaccines.

Live bacterial vaccines are conventionally administered parenterally, by injection or in oral formulation. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 10-95% of active ingredient, preferably 25-70%.

The vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective and immunogenic. The quantity to be administered depends on the subject to be treated, including, e.g., the capacity of the individual's immune system to synthesize antibodies, and the degree of protection desired. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner. However, suitable dosage ranges are of the order of several hundred micrograms active ingredient per vaccination. Suitable regimens for initial administration and booster shots are

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also variable but are typified by an initial administration followed by subsequent inoculations or other administrations.

5           The manner of application may be varied widely. Any of the conventional methods for administration of a vaccine are applicable. These are believed to include oral application on a solid physiologically acceptable base or in a physiologically acceptable dispersion,  
10          parenterally, by injection or the like. The dosage of the vaccine will depend on the route of administration and will vary according to the size of the host.

          In many instances, it will be desirable to have  
15          multiple administrations of the vaccine, usually not exceeding six vaccinations, more usually not exceeding four vaccinations and preferably one or more, usually at least about three vaccinations. The vaccinations will normally be from two to twelve week intervals, more  
20          usually from three to five week intervals. The course of the immunization may be followed by assays for antibodies for the supernatant antigens. The assays may be performed by labeling with conventional labels, such as radionuclides, enzymes, fluorescers, and the like. These  
25          techniques are well known and may be found in a wide variety of patents, such as U.S. Patent Nos. 3,791,932; 4,174,384 and 3,949,064, as illustrative of these types of assays.

30          The invention also contemplates the use of disclosed nucleic acid segments in the construction of expression vectors or plasmids and use in host cells. The following is a general discussion relating to such use and the particular considerations in practicing this aspect of  
35          the invention.

Host Cell Cultures and Vectors

In general, of course, prokaryotes are preferred for the initial cloning of DNA sequences and constructing the  
5 vectors useful in the invention. For example, in addition to the particular strains mentioned in the more specific disclosure below, one may mention by way of example, strains such as *E. coli* K12 strain 294 (ATCC No. 31446), *E. coli* B, and *E. coli* X 1776 (ATCC No. 31537).  
10 These examples are, of course, intended to be illustrative rather than limiting.

Prokaryotes are also preferred for expression. The  
aforementioned strains, as well as *E. coli* W3110 (F-,  
15 lambda-, prototrophic, ATCC No. 273325) or other enterobacteriaceae such as *Salmonella typhimurium* or *Serratia marcesans*, and various *Pseudomonas* species may be used.

In general, plasmid vectors containing replicon and  
20 control sequences which are derived from species compatible with the host cell are used in connection with these hosts. The vector ordinarily carries a replication site, as well as marking sequences which are capable of  
25 providing phenotypic selection in transformed cells. For example, *E. coli* is typically transformed using pBR322, a plasmid derived from an *E. coli* species (see, e.g., Bolivar et al., 1977). The pBR322 plasmid contains genes  
30 for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells. The pBR plasmid, or other microbial plasmid or phage must also contain, or be modified to contain, promoters which can be used by the microorganism for expression.

35 Those promoters most commonly used in recombinant DNA construction include the lactose promoter systems (Chang et al., 1978; Itakura et al., 1977; Goeddel et

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al., 1979) and a tryptophan (trp) promoter system (Goeddel et al., 1979; EPO Appl. Publ. No. 0036776). While these are the most commonly used, other microbial promoters have been discovered and utilized, and details  
5 concerning their nucleotide sequences have been published, enabling a skilled worker to ligate them functionally with plasmid vectors (Siebwenlist et al., 1980). Certain genes from prokaryotes may be expressed efficiently in *E. coli* from their own promoter sequences,  
10 precluding the need for addition of another promoter by artificial means.

The following examples are intended to illustrate the practice of the present invention and are not  
15 intended to be limiting. Although the invention is here demonstrated with  $\beta$ -lactamase expressed on the surface of a cell membrane, numerous other proteins with various functions could be similarly expressed. These would include polypeptides with catalytic functions, metal  
20 binding capability and specific binding activity toward cell receptor sites. Moreover, the expression vectors and chimeric genes used therein may be constructed from a wide variety of targeting and transversing sequences, and are not limited to those derived from OmpA and Lpp.  
25

#### EXAMPLE 1

The following example illustrates the construction  
30 of a recombinant vector encoding a desired protein, targeting and membrane translocating sequences. When used to transform suitable bacterial hosts, such a vector enables surface expression of active proteins, as shown here for the production of  $\beta$ -lactamase.  
35



Construction of Plasmid pTX101 Containing Tripartite  
Chimeric Gene for  $\beta$ -Lactamase Expression

Plasmid pTX101 was prepared from pJG311, which  
5 contains the signal sequence and first 9 N-terminal amino  
acids of the mature major outer membrane lipoprotein of  
*E. coli* and the complete  $\beta$ -lactamase sequence. A unique  
*EcoRI* site in the linker region between the peptide  
region and the  $\beta$ -lactamase was cut with *EcoRI*. The cut  
10 plasmid was isolated from a low melting point agarose gel  
and the ends were made blunt using the Klenow fragment.  
pRD87, containing the *OmpA* gene, was simultaneously cut  
with *HpaI* and *SphI*, both unique sites, generating a 342  
bp fragment containing the sequence for five of the eight  
15 outer membrane spanning domains of *OmpA*. This fragment  
was isolated from a low melting point agarose gel and  
made blunt with T4 DNA polymerase. The fragment, coding  
for amino acid residues 46-159 of *OmpA*, was ligated to  
the above pJG311 vector to make pTX101, which codes for  
20 the *Lpp-OmpA- $\beta$ -lactamase* fusion. The ligation was  
transformed into *E. coli* strain JM109 made competent by  
the rubidium chloride method.

The *Lpp-OmpA- $\beta$ -lactamase* was expressed from the  
25 strong *lpp* promoter which is inducible by IPTG (isopropyl  
thiogalactoside). Plasmid pTX101 also carries the *lacI*  
repressor. Although induction with IPTG resulted in high  
levels of protein production which are lethal to the  
cell, good expression was nevertheless obtained in the  
30 absence of inducer. Cultures were harvested in late  
exponential phase and the cells lysed and separated into  
soluble and cell envelope fractions by high speed  
centrifugation. Approximately 84% of total  $\beta$ -lactamase  
activity from JM109(pTX101) lysates was found in the cell  
35 envelope fraction. Essentially all the remaining  
activity was present in the soluble fraction of the cell  
lysates with less than 0.5% in the extracellular fluid.

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Even after prolonged incubation of stationary phase cells (24 hrs) there was no increase in the percentage of  $\beta$ -lactamase in the extracellular fluid, indicating that the fusion protein was not released from the cells, Figure 5.

5 Qualitatively similar results were observed when the distribution of the fusion protein in the different fractions was examined by immunoblotting with  $\beta$ -lactamase or OmpA-specific antisera. The Lpp- $\beta$ -lactamase protein from plasmid pJG311 had 2-fold higher total activities  
10 compared to the three-part fusion which contained the OmpA insert. This protein was also found predominantly in the membrane pellet (98% of total activity).

## EXAMPLE 2

15

This example illustrates that a heterologous polypeptide prepared as in Example 1 is exposed to the external medium and retains activity.

### 20 Cell Fractionation

First it was demonstrated that the fusion protein was localized to the outer membrane. Cells were harvested from 200 ml of LB medium containing 0.2%  
25 glucose at an  $A_{600}=1.0$ , washed in 25 mM Tris-HCl (pH 7.4) and resuspended in 10 ml of the same buffer containing 1 mM EDTA and 100  $\mu$ g/ml lysozyme at 4°C. After a 2 minute incubation the cells were lysed by two passages through a French pressure cell at 10,000 psi. The cellular debris  
30 was removed by centrifugation at 2,500 xg for 8 minutes and the total membranes were spun down by centrifugation at 115,000 xg for 1 hour. Membranes were resuspended in 0.8 ml of Tris-HCl buffer containing 25% sucrose and loaded onto a step gradient of 20, 35, 40, 45, 50 and 55%  
35 (w/w) sucrose. After centrifugation at 165,000 xg for 16 hours in a Beckman SW41Ti rotor, 0.5 ml fractions were collected from the bottom of the tube. The density of

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the fractions was determined from refractive index measurements. The concentration of sucros was lowered to <10% (w/w) by diluting the sampl s with Tris-HCl buffer followed by centrifugation to pellet the membranes.

Two distinct protein peaks were obtained in fractions having the expected densities for inner and outer membrane vesicles (Osborn et al., 1972). Virtually all the  $\beta$ -lactamase enzymatic activity was found in the higher density fractions which corresponded to the outer membrane vesicles, Figure 2. A protein band migrating at a molecular weight of approximately 43,000 daltons, the expected size of Lpp-OmpA- $\beta$ -lactamase band was found and it was comparable in abundance to that of the major outer membrane proteins, Figure 3A. The fusion protein was subjected to some degradation resulting in the appearance of lower molecular weight bands that crossreacted with  $\beta$ -lactamase-specific antibodies in immunoblots, Figure 3B. The relatively small proportion of degradation fragments indicated that most of the fusion protein was not subjected to proteolysis.

#### Exposure of $\beta$ -Lactamase on Cell Surface

Localization of the  $\beta$ -lactamase domain with respect to the external surface of *E. coli* was determined by various immunocytochemistry methods, activity assays and protease accessibility experiments. For immunofluorescence determinations, whole cells were labelled with rabbit  $\beta$ -lactamase-specific antibodies followed by secondary rhodamine-conjugated goat anti-rabbit antibodies. Washed, mid-exponential phase cells were resuspended in phosphate buffered saline (PBS) with or without 0.1 mg/ml trypsin and incubat d at 37°C. Soyban trypsin inhibitor was add d at different times to stop the reaction and incubation at 37°C was continued

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for a total of 1 hour. All subsequent procedures were conducted at room temperature. The cells were washed with PBS, incubated for 45 minutes with PBS and 1% bovine serum albumin, washed and then incubated in the same  
5 PBS/BSA solution with rabbit anti- $\beta$ -lactamase antibodies at 1:1,000 dilution for 45 minutes. Following another three washes with PBS/BSA, the cells were mixed with rhodamine-conjugated goat anti-rabbit antibodies, incubated for 45 minutes and then washed three more  
10 times. Finally, the cells were resuspended in PBS and examined by phase contrast and video enhanced fluorescence microscope.

In control experiments, no fluorescence above  
15 background was detectable with JM109(pJG311) cells expressing the Lpp- $\beta$ -lactamase fusion protein, indicating that there was no exportation to the outer surface. Figures 4A and 4B show a comparison of the same field of JM109(pTX101) cells viewed with fluorescence phase  
20 contrast microscopies. Nearly all the cells became fluorescent, indicating sequences recognized by the anti- $\beta$ -lactamase antibodies. Incubation with trypsin for various times prior to antibody labelling resulted in a gradual decrease in the fluorescent signal. After 1 hour  
25 of incubation no signal was detected.

For immunoelectron microscopy cells were labelled with rabbit anti- $\beta$ -lactamase specific antibodies, washed in various buffers as described above for the  
30 immunofluorescence experiments and reacted with secondary 30 nm diameter colloidal gold conjugated goat anti-rabbit antibodies. The labelled cells were positively stained with uranyl-acetate and viewed by scanning electron  
microscopy. In control experiments, no labelling  
35 occurred with JM109(pJG311) cells. Figure 9 shows JM109(pTX101) cells so labelled, indicating the presence

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of sequences recognized by the anti- $\beta$ -lactamas antibodies on the external surface.

5 The presence of enzymatically activ  $\beta$ -lactamase on the cell surface was determined from protease accessibility experiments and the rates of hydrolysis of substrates not readily diffusible through the outer membrane. Cultures grown in M9 medium were harvested at  $A_{600}=1.0$ , washed with fresh medium and resuspended in M9 salts without glucose or antibiotics. The  $\beta$ -lactamase activity in the whole cells was determined using nitrocefin and penicillin G as substrates. The cells were incubated for 1 hr at 37°C in the presence or absence of 0.1 mg/ml of either proteinase K or trypsin. 10 The protease digestions were stopped by adding 10 mM phenylmethylsulfonyl fluoride or 0.2 mg/ml soybean trypsin inhibitor respectively. Subsequently, the cells were lysed and centrifuged at 2500 xg for 8 min to remove unbroken cells. The membranes were pelleted as described above, resuspended in 50 mM potassium phosphate buffer, pH 6.5 and the remaining enzymatic activity measured. 20

In cells containing plasmid pTX101, approximately 20% of the total membrane-bound activity was reproducibly lost after a one hour incubation with either trypsin or proteinase K, compared with only a 3% decrease in JM109(pJG311), Table 1. A comparable, somewhat higher percentage of surface exposed activity was obtained from the rates of hydrolysis of nitrocefin in intact and lysed cells. Nitrocefin does not cross the outer membrane of *E. coli* and therefore can be used to test activity of extracellular  $\beta$ -lactamase (Kornacker and Pugsley, 1990). The rate of hydrolysis of nitrocefin by intact cultures of JM109(pJG311) was in agreement with results of protease accessibility studies, indicating Lpp- $\beta$ -lactamase is not transported across the cell surface. 35 Approximately 20-30% of the enzymatic activity of Lpp-

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OmpA- $\beta$ -lactamase was surface exposed on cells grown at 37°C, a significant increase over the background in control cultures, see Table 1.

5

TABLE 1

10

Percent Surface Exposed  $\beta$ -Lactamase as Determined by Protease Accessibility and Enzymatic Activity Using Nitrocefin

15

20

Percent decrease in penicillin G hydrolysis following incubation with:				Nitrocefin activity in intact cells as percentage of total activity in membranes <sup>b</sup>
Plasmid	Temperature	Proteinase K <sup>a</sup>	Trypsin <sup>a,b</sup>	
pJG311	37°C	3	3	6
pTX101	37°C	23	18	38
pTX101	24°C		89	81

25

<sup>a</sup> Cells were incubated with proteinase K or trypsin for one hour and the total membrane fractions were isolated as described in the materials and methods sections. The percent of exposed  $\beta$ -lactamase corresponds to the activity remaining after incubation with proteases relative to untreated cells.

30

<sup>b</sup> The standard deviation for all experiments was less than  $\pm 5\%$  of the reported mean values.

35

EXAMPLE 3

40

The following example demonstrates that even after extended incubation, the region of the tripartite fusion containing the target protein remains stably anchored to the outer membrane of the host cell. This example demonstrates the surface stability of surface-expressed fusion polypeptides using the disclosed methods.

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Surface Stability of Translocated Fusion Protein  $\beta$ -Lactamase

5 JM109 cells with the plasmid pTX101 were grown in LB supplemented with glucose and ampicillin. After 4, 6, 8 and 24 hours, 10 ml samples were collected and separated into culture supernatant, soluble and membrane fractions, Figure 5. The cells were first pelleted by centrifugation at 8,000 xg and the resulting supernatant was saved as the culture supernatant fraction. The pelleted cells were resuspended in 50 mM potassium phosphate buffer (pH 7) and lysed in a French pressure cell at 20,000 psi. The lysed samples were centrifuged at 2,500 xg to pellet any unlysed cells and then 10 centrifuged for 1 hr at 115,000 xg. The supernatant from the high speed centrifugation was removed and saved as the soluble fraction and the pelleted membranes resuspended in 50 mM potassium phosphate buffer to obtain the membrane fractions.  $\beta$ -lactamase activity performed 15 on the fractions indicated that even after prolonged incubation (24 hrs) the fusion was stably anchored to the outer membrane (Figure 5).

EXAMPLE 4

25 This example illustrates that efficient surface-expression and maintenance of activity of surface-expressed polypeptides is affected by the culture temperature. The example is illustrated with  $\beta$ -lactamase, but effective surface expression with 30 maintenance of function is also affected by temperature for alkaline phosphatase.

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Effect of Temperature on  $\beta$ -Lactamase Expression and Activity

5 Cultures grown at 24°C exhibited almost quantitative  $\beta$ -lactamase activity on the cell surface. The rate of nitrocefin hydrolysis and trypsin accessibility indicated 80-87% surface exposure, Table 1.

EXAMPLE 5

10

The following example illustrates expression of alkaline phosphatase on the outer membrane surface of *E. coli*. Alkaline phosphatase is a large dimeric enzyme with a monomer size of approximately 43,000 D. Disulfide bonds form rapidly after the protein has been exported from the cytoplasm. The expression of active protein on the bacterial surface indicates that there is no significant effect on the protein's ability to retain or fold to its native form after membrane translocation.

15 This example also illustrates the versatility of the method in that alkaline phosphatase is a relatively large protein. In this example, tertiary and quaternary structures are unaffected by the expression.

25 Expression of Alkaline Phosphatase on *E. coli* Cell Surface

Plasmid pTX101 was cut with *EcoRI* at the unique site in the linker region between the *OmpA* and  $\beta$ -lactamase.

30 Subsequently the DNA was treated with the Klenow fragment of DNA polymerase to create blunt ends. The *phoA* gene coding for the sequence of alkaline phosphatase was isolated from the plasmid pSWFII. A DNA fragment containing the *phoA* gene was obtained by cutting pSWFII

35 with *SbaI* and then blunt-ended using the Klenow fragment. The linearized pTX101 vector and the *phoA* gene fragment were ligated overnight and the DNA was transformed into



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*E. coli* strain JM109. The new plasmid encoding the Lpp-OmpA-PhoA tripartite fusion was designated pTX1000. Exposure of the alkaline phosphatase on the surface of *E. coli* was tested by immunofluorescence microscopy using anti-alkaline phosphatase antibodies.

The degree of localization of alkaline phosphatase on the cell surface is expected to be enhanced in the strain JCB572 (Bardwell et al., 1991) which is deficient in the gene for the *E. coli* periplasmic protein disulfid isomerase, DsbA, and in cultures incubated at sub-optimal growth temperatures, for example 24°C.

#### PROPHETIC EXAMPLE 6

The present example outlines the procedure contemplated as useful for expressing an antibody on the surface of *E. coli*. The antibody used for illustration is a catalytic antibody capable of catalysis in addition to binding its cognate antigen. Although this example is illustrated with antibody 37C4 against a particular hapten, other high affinity antibodies could be surface-expressed in a like manner. The example shows how the disclosed methods could be used to prepare a single-chain  $F_v$ , that is, a recombinant protein composed of a  $V_L$  chain linked to a  $V_H$  chain with a polypeptide linker. This particular  $ScF_v$  is a catalytic antibody.

#### Expression of Single-Chain $F_v$ Antibody on *E. coli* Cell Surface

Antibody 37C4 exhibits high binding against the hapten tris(4-methoxyphenyl)phosphonium (dissociation constant  $>10^{-10}M^{-1}$ ). The antibody acts as a catalyst for the cleavage of various trityl ethers, increasing the reaction rate by about 200 fold compared to the uncatalyzed reaction in the absence of antibody. Total

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mRNA from the 37C4 hybridoma line is isolated and purified by standard techniques (Ausubel et al. 1987). The purified mRNA is used as a template for cDNA synthesis using a polymerase chain amplification technique (Sastry et al. 1989). The  $V_L$  and the  $V_H$  domains of the 37C4 antibody are cloned using suitable primers designed to introduce an in-frame *EcoRI* restriction site at the N-terminus of the  $V_H$  and another one at the carboxy terminus of the  $V_L$  for easy subcloning of the  $F_V$  gene into the surface expression vector pTX101. Plasmid pTX101 contains a unique *EcoRI* site located at the downstream end of the DNA sequence for the OmpA domain and immediately before the beginning of the  $\beta$ -lactamase gene. An Lpp-OmpA-scF<sub>V</sub> tripartite fusion is constructed by digestion of pTX101 with *EcoRI* and ligation of the scF<sub>V</sub> fragment. The resulting plasmid is transformed into *E. coli* strain JM109. The presence of the single-chain antibody on the cell surface allows the cells to bind to a complex of tris(4-methoxyphenyl)phosphonium antigen linked to the protein avidin. The antigen is linked to avidin via its carboxy terminus by standard techniques (Staros et al. 1986). Finally, avidin is detected by immunofluorescence microscopy using anti-avidin antibodies conjugated to fluorescein (obtained from Vector Laboratories, Burlingame, CA. Cells expressing the scF<sub>V</sub> fragment give a fluorescence signal whereas control *E. coli* do not.

#### PROPHETIC EXAMPLE 7

30

The present example outlines the procedure contemplated as useful for the selection of antibodies with high antigen binding affinity. The method is based on a selection procedure for recombinant antibody fragments on the surface of *E. coli*.

35

-40-

The method illustrated will overcome many of the problems currently associated with display of antibody molecules on phage surfaces. In particular, subcloning will not be necessary for production, the number of antibodies on the cell surface can be controlled and the greater flexibility in the design of the expression system will help ensure proper folding.

Selection of High Antigen Binding Affinity Antibodies  
10 Using a Cell Surface Display System

BALB/C female mice (6-8 weeks old) are immunized with the hapten tris(4-methoxyphenyl)phosphonium coupled to Supercarrier (Pierce Chemical, Chicago, IL) dissolved in Freund's complete adjuvant administered intraperitoneally at a dose of 1 mg per animal. Follow up injections are given intramuscularly once per week for three weeks, rested 2 weeks and given a booster shot before checking for antibody production. Incomplete Freund's adjuvant is used for all injections subsequent to the first injection.

A library of single-chain  $F_v$  antibodies is constructed using a polymerase chain reaction with total spleen mRNA from the immunized mice (Clarckson et al., 1991). The PCR primers are designed to introduce an in-frame *EcoRI* restriction site at the N-terminus of the  $V_H$  and another restriction site at the carboxy terminus of the  $V_L$  for easy subcloning of the  $F_v$  gene into the surface expression vector pTX101. Subsequently, the library DNA is digested with *EcoRI*, a gene fragment of approximately 730 base pairs containing the entire sc $F_v$  gene is identified. This DNA fragment is isolated and ligated to *EcoRI* digested plasmid pTX101. The ligation mixture is transformed into competent *E. coli* cells and transformants are selected on LB plates containing the antibiotic chloramphenicol. Plasmids in which the sc $F_v$

-41-

is inserted in the correct orientation result in expression of tripartite fusion proteins in the order (from the amino terminus): Lpp-OmpA-scF<sub>v</sub>. Colonies are pooled from the plate and grown in rich media at 23°C to  
5 allow efficient localization of the scF<sub>v</sub> to the cell surface. Approximately 10<sup>9</sup> cells are diluted in buffer to halt further growth and are loaded onto an affinity column (approx 5 ml bed vol) containing the immobilized hapten tris(4-methoxyphenyl)phosphonium. The column is  
10 washed with Tris-HCl buffer, pH 7.0. Bound cells are eluted by applying a linear gradient of the hapten. Elution of the cells from the column is directly related to the binding affinity of the exposed antibodies. This results in enrichment of high-binding affinity antibodies  
15 expressed on the surface of cells. The cells are collected, grown and used to prepare antibodies.

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CLAIMS

1. A tripartite chimeric gene for expression of a desired polypeptide comprising:

5

a targeting DNA sequence encoding a polypeptide capable of targeting a fusion polypeptide to a gram-negative cell outer membrane;

10

a DNA segment encoding a transmembrane amino acid sequence capable of transporting a heterologous or a homologous polypeptide through a gram-negative outer membrane; and

15

at least one DNA segment encoding a heterologous or a soluble homologous polypeptide

wherein the chimeric gene is expressible in a gram negative bacterial cell when preceded by a functional promoter,

20

or

~~A recombinant vector capable of expressing a~~  
25 ~~heterologous or a soluble homologous polypeptide on a~~  
~~gram-negative bacterium outer membrane external surface,~~  
the vector comprising

30

a targeting DNA sequence encoding a polypeptide capable of targeting to the outer membrane of a gram negative bacterial cell;

35

a transmembrane DNA sequence positioned adjacent to the targeting DNA sequence, said transmembrane sequenc encoding a polypeptide capable of transversing the cell outer membrane; and

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a DNA sequence encoding a heterologous or soluble homologous polypeptide, said sequence being positioned adjacent to said transmembrane sequence.

5

2. The recombinant vector of claim 1 wherein the targeting DNA for an outer membrane protein precedes the transmembrane sequence for said membrane protein.

10

3. The chimeric gene or the recombinant vector of claim 1 wherein the targeting DNA sequence encodes an amino acid sequence consisting of a signal sequence and at least part of an outer membrane protein amino acid sequence from a gram-negative bacterium.

15

4. The chimeric gene or the recombinant vector of claim 1 further comprising a signal sequence having at least a part of an amino acid sequence from an outer membrane lipoprotein of *E. coli*.

20

5. The chimeric gene or recombinant vector of claim 1 wherein the outer membrane protein amino acid sequence comprises at least a part of *OsmB*, *TraT*, *NlpB*, *BlaZ*, *OmpA*, *LamB*, *OmpC*, *PhoE* or *Pseudomonas* lipoprotein 1.

25

6. The chimeric gene or the recombinant vector of claim 1 wherein the targeting DNA sequence encodes N-terminal amino acid residues derived from nucleic acid of Figure 7 (SEQ ID NO:1) at base pair positions 1-87.

30

35

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7. The chimeric gene or the recombinant vector of claim 1 wherein the encoded transmembrane protein sequence comprises at least part of an outer membrane protein of PhoE, LamB, OmpC, OmpF, OmpT, pilin or enterobactin receptor or OmpA.

8. The chimeric gene or the recombinant vector of claim 1 wherein the transmembrane sequence comprises an amino acid sequence derived from nucleic acid sequence of Figure 7 (SEQ ID NO:1) at base pairs 94-435.

9. The chimeric gene or the recombinant vector of claim 1 wherein the gram negative cell is *Escherichia*, *Salmonella*, *Klebsiella*, *Erwinia*, *Shigella*, *Serratia* or *Vibrio*.

10. The chimeric gene or the recombinant vector of claim 1 wherein the gram negative cell is a mutant *E. coli* or *Salmonella* characterized as having an impaired ability to form native disulfide bonds.

11. The chimeric gene or the recombinant vector of claim 1 wherein the soluble homologous polypeptide is  $\beta$ -lactamase or alkaline phosphatase.

12. The chimeric gene or the recombinant vector of claim 1 wherein the heterologous polypeptide is a single chain antibody or antibody fragment.

13. An *Escherichia coli* or *Salmonella* transformant prepared by transformation with the vector of claim 2.

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14. A method for obtaining a polypeptide anchored on the outer membrane surface of a gram-negative bacterial host cell comprising the steps:

- 5           preparing a recombinant vector according to claim 1;  
transforming a gram-negative bacterial cell with the recombinant vector;  
10          culturing the transformed cells;  
screening for transformants; and  
culturing the transformant to obtain cells having a  
15          polypeptide anchored on the outer membrane surface.

15. A method of eliciting an immune response in a  
20          mammal, comprising the steps:

- a) selecting a polypeptide toward which an immune response is desired;  
25          b) preparing a vector in accordance with claim 1 wherein the heterologous or soluble homologous polypeptide is an antigenic polypeptide from an infectious agent; and  
30          c) administering to a mammal a pharmaceutically acceptable preparation obtained from admixing cells transformed with the vector of step (b) with a vehicle suitable for administration in a mammal.

35

16. A heterologous or soluble homologous polypeptide expressed by a gram-negative cell transformed with a

-50-

recombinant vector comprising the chimeric gene of claim 1.

5 17. A kit useful for transforming gram-negative bacterial host cells comprising:

10 a transporter being compartmentalized to receive one or more container means in close confinement therein; and

15 a first container means comprising at least one recombinant vector in accordance with claim 1, the vector being suitably aliquoted into the container.

20 18. The kit of claim 17 wherein the vector is in accordance with SEQ ID NO:1.

19. A recombinant vector in accordance with SEQ ID NO: 1.

25

20. A method for removing contaminants from fluids comprising admixing a gram-negative bacterium transformed with the recombinant vector of claim 1 with a contaminant-containing fluid wherein the encoded  
30 heterologous or soluble homologous polypeptide located on the bacterial surface binds selectively with the contaminant.

35 21. The method of claim 20 wherein the encoded polypeptide is metallothionein or a single chain antibody or antibody fragment.

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22. The method of claim 20 further comprising cross-linking the transformed bacterial cells before admixing with the contaminant.

5

23. An immobilized enzyme system comprising an enzyme expressed on the outer membrane surface of a gram-negative bacterial host cell transformed with the recombinant vector of claim 1 wherein the encoded  
10 heterologous or soluble homologous polypeptide comprises a biocatalytically active polypeptide.

24. The immobilized enzyme system of claim 23 wherein  
15 the encoded polypeptide comprises a catalytic antibody.

25. A method of selecting high affinity recombinant antibodies or antibody fragments, comprising the steps:  
20

- a) constructing a library of genes encoding at least one antibody or antibody fragment;
- b) preparing vectors in accordance with claim 1  
25 wherein the heterologous or soluble homologous polypeptide comprises one or more genes of said library;
- c) transforming a gram-negative bacterial cell using  
30 the recombinant vectors;
- d) culturing said transformed cells; and
- e) binding the expressed antibodies or antibody  
35 fragments to a suitable antigen wherein cells expressing high affinity antibodies or antibody fragments are selected.

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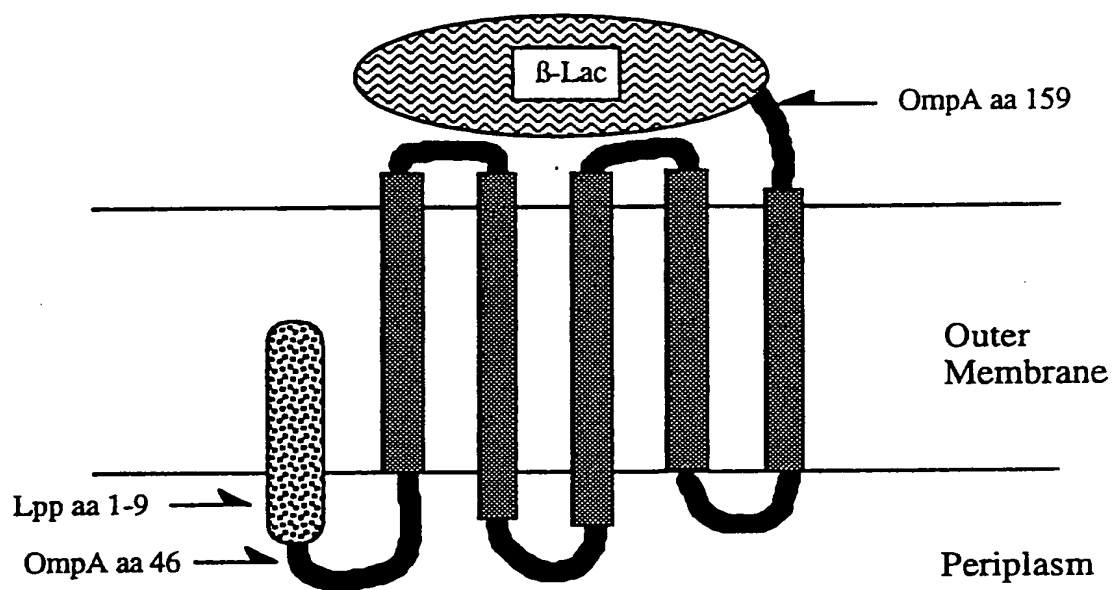


FIG. 1

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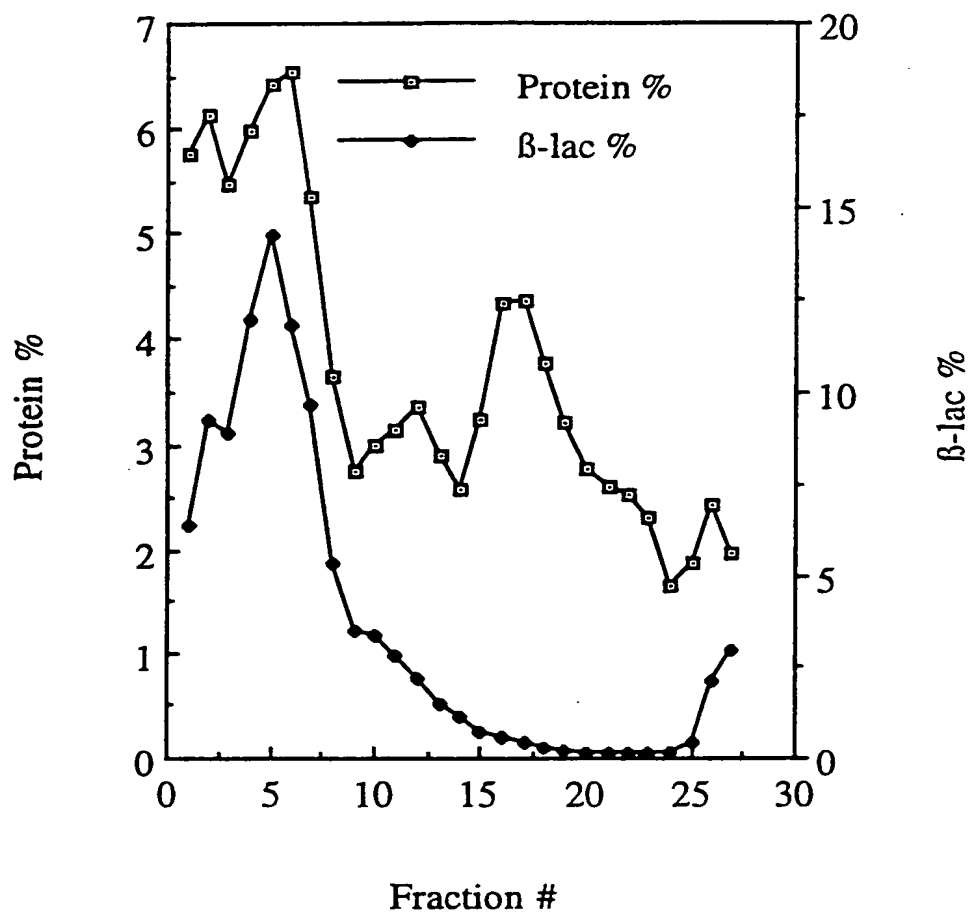


FIG. 2

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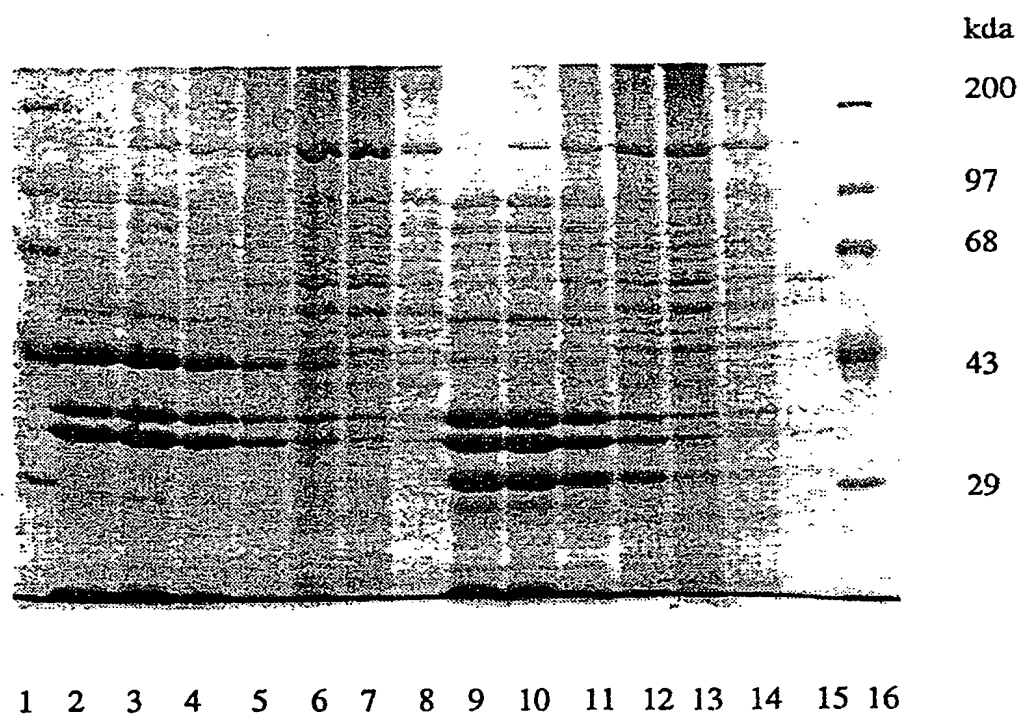


FIG. 3A

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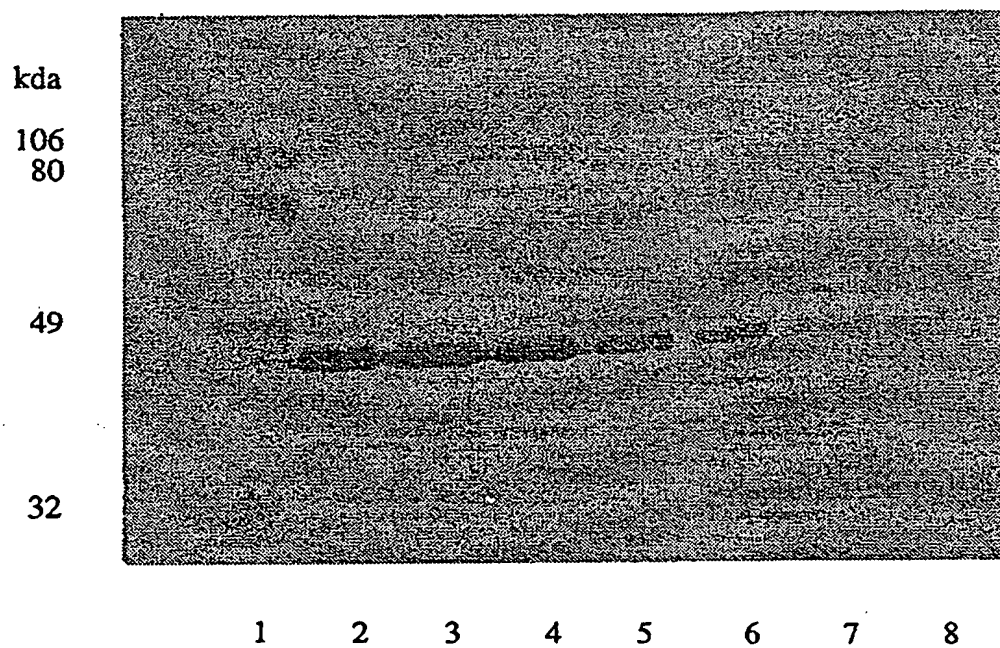


FIG. 3B

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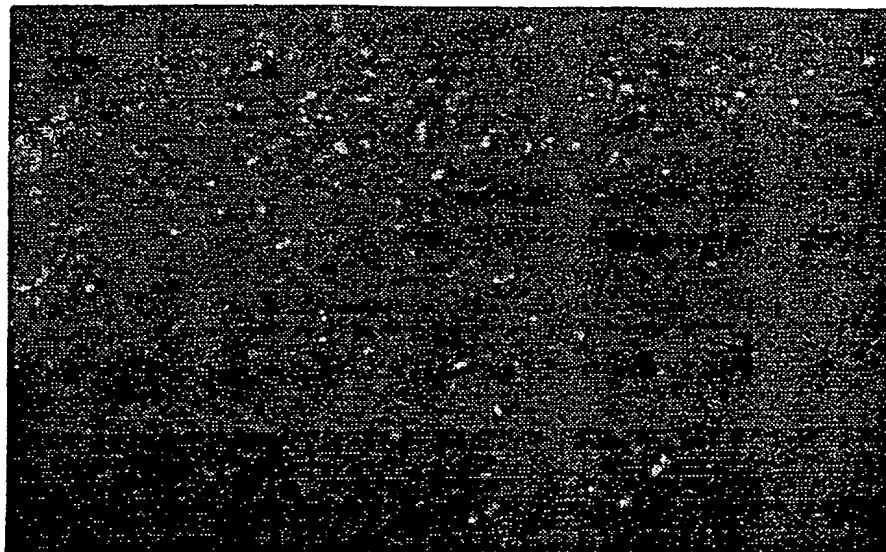


FIG. 4A

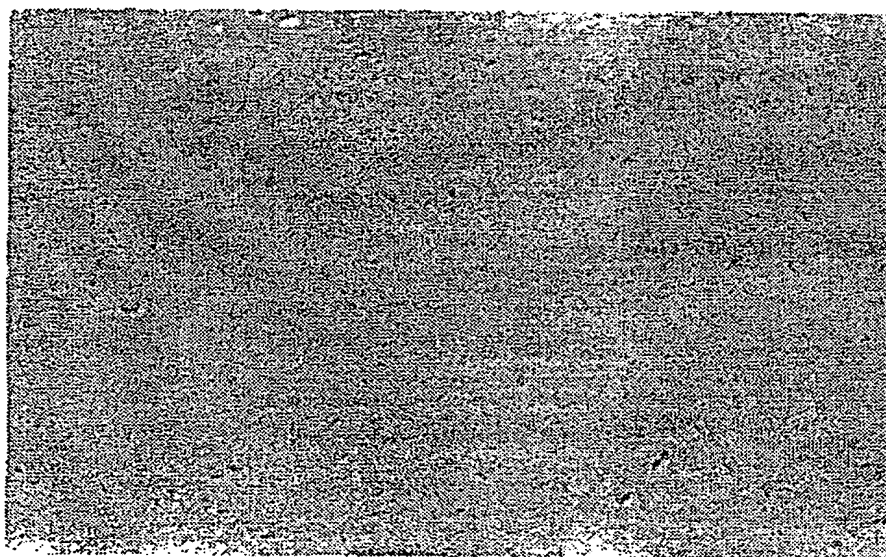


FIG. 4B

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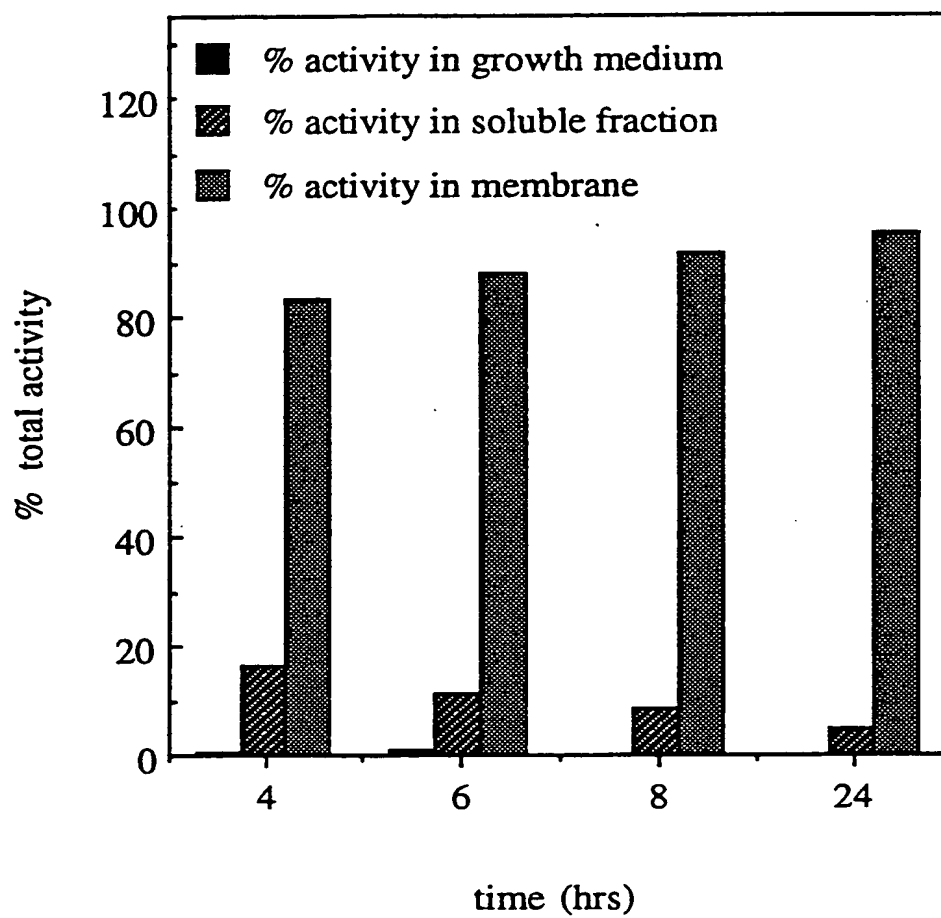


FIG. 5

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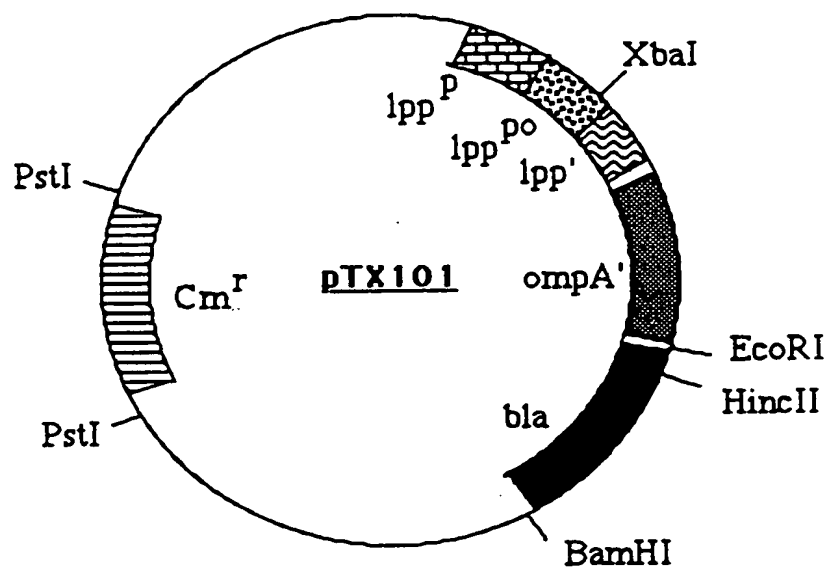


FIG. 6

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FIG. 7A

ATGAAAGCTA CTAAACTGGT ACTGGGCGG GTAATCCTGG GTTCTACTCT GCTGGCAGGT 60  
TGCTCCAGCA ACGCTAAAAT CGATCAGGGA ATTAACCCGT ATGTTGGCTT TGAAATGGGT 120  
TAGGACTGGT TAGGTCGTAT GCCGTACAAA GGCAGCGTTG AAAACGGTGC ATACAAAGCT 180  
CAGGGCGTTC AACTGACCGC TAAACTGGGT TACCCAATCA CTGACGACCT GGACATCTAC 240  
ACTCGTCTGG GTGGCATGGT ATGGCGTGCA GACACTAAAT CCAACGTTTA TGGTAAAAAC 300  
CAGCACACCG GCGTTTCTCC GGTCTTCGCT GCGGGTGTG AGTACGGGAT CACTCCTGAA 360  
ATCGCTACCC GTCTGGAATA CCAGTGGACC AACAAACATCG GTGACGCACA CACCATCGGC 420  
ACTCGTCCGG ACAACGGAAT TCCGGGTAC CCAGAAACGC TGGTGAAAGT AAAAGATGCT 480  
GAAGATCAGT TGGGTGCAG AGTGGGTTAC ATCGAACTGG ATCTCAACAG CGGTAAGATC 540  
CTTGAGAGTT TTCGCCCCGA AGAACGTTTT CCAATGATGA GCACTTTTAA AGTTCTGCTA 600  
TGTGGCGCGG TATTATCCCG TGTGACGCC GGGCAAGAGC AACTCGGTG CCGCATACAC 660  
TATTCTCAGA ATGACTTGGT TGAGTACTCA CCAGTCACAG AAAAGCATCT TACGGATGGC 720  
ATGACAGTAA GAGAATTATG CAGTGCTGCC ATAACCATGA GTGATAACAC TCGGGCCAAC 780  
TTACTTCTGA CAACGATCGG AGGACCGAAG GAGCTAACCG CTTTTTTGCA CAACATGGGG 840  
GATCATGTAA CTCGCCTTGA TCGTTGGGAA CCGGAGCTGA ATGAAGCCAT ACCAAACGAC 900  
GAGCGTGACA CCACGATGCC TGCAGCAATG GCAACAACGT TCGGCAAACT ATTAACCTGG 960  
GAACTACTTA CTCTAGCTTC CCGGCAACAA TTAATAGACT GGATGGAGGC GGATAAAGTT 1020

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FIG. 7B

GCAGGACCAC TTCTGGGCTC GCCCCTTCCG GCTGGCTGGT TTATTCGTGA TAAATCTGGA 1080  
GCCGGTGAGC GTGGGTCTCG CGGTATCATTT GCAGCACTGG GGCACAGATGG TAAGCCCCCTCC 1140  
CGTATCGTAG TTATCTACAC GACGGGGAGT CAGGCAACTA TGGATGAACG AAATAGACAG 1200  
ATCGCTGAGA TAGGTGCCTC ACTGATTAAG CATTTGGTAAC TGTCAGACCA AGTTTACTCA 1260  
TATATACTTT AGA 1273

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FIG.8A

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ATG AAA GCT ACT AAA CTG GTA CTG GGC GCG GTA ATC CTG GGT TCT ACT CTG 51
Met Lys Ala Thr Lys 5 Leu Val Ile Leu Gly Ser Thr Leu 15
1
CTG GCA GGT TGC TCC AGC AAC GCT AAA ATC GAT CAG GGA ATT AAC CCG TAT 102
Leu Ala Gly Cys Ser Ser Asn Ala Lys 25 Ile Asp Gln Gly 30 Ile Asn Pro Tyr
20
GTT GGC TTT GAA ATG GGT TAC GAC TGG TTA GGT CGT ATG CCG TAC AAA GGC 153
Val Gly Phe Glu Met 40 Gly Tyr Asp Trp Leu Gly Arg Met Pro Tyr Lys Gly 50
35
AGC GTT GAA AAC GGT GCA TAC AAA GCT CAG GGC GTT CAA CTG ACC GCT AAA 204
Ser Val Glu Asn Gly 55 Ala Tyr Lys Ala Gln Gly Val Gln Leu Thr Ala Lys 65
60
CTG GGT TAC CCA ATC ACT GAC GAC CTG GAC ATC TAC ACT CGT CTG GGT GGC 255
Leu Gly Tyr Pro Ile Thr 75 Asp Asp Leu Asp Ile Tyr Thr Arg Leu Gly Gly 85
70
ATG GTA TGG CGT GCA GAC ACT AAA TCC AAC GTT TAT GGT AAA AAC CAC GAC 306
Met Val Trp Arg Ala Asp Thr Lys Ser Asn Val Tyr Gly Lys Asn His Asp 95
90
100

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FIG. 8B

ACC GGC GTT TCT CCG GTC TTC GCT GGC GGT GAG TAC GCG ATC ACT CCT 357  
 Thr Gly Val Ser Pro Val Phe Ala Gly Gly Val Glu Tyr Ala Ile Thr Pro  
 105 110 115

GAA ATC GCT ACC CGT CTG GAA TAC CAG TGG ACC AAC AAC ATC GGT GAC GCA 408  
 Glu Ile Ala Thr Arg Leu Glu Tyr Gln Trp Thr Asn Asn Ile Gly Asp Ala  
 120 125 130 135

CAC ACC ATC GGC ACT CGT CCG GAC AAC GGA ATT CCG GGT CAC CCA GAA ACG 459  
 His Thr Ile Gly Thr Arg Pro Asp Asn Gly Ile Pro Gly His Pro Glu Thr  
 140 145 150

CTG GTG AAA GTA AAA GAT GCT GAA GAT CAG TTG GGT GCA CGA GTG GGT TAC 510  
 Leu Val Lys Val Lys Asp Ala Glu Asp Gln Leu Gly Ala Arg Val Gly Tyr  
 155 160 165 170

ATC GAA CTG GAT CTC AAC AGC GGT AAG ATC CTT GAG AGT TTT CGC CCC GAA 561  
 Ile Glu Leu Asp Leu Asn Ser Gly Lys Ile Leu Glu Ser Phe Arg Pro Glu  
 175 180 185

GAA CGT TTT CCA ATG ATG AGC ACT TTT AAA GTT CTG CTA TGT GGC GCG GTA 612  
 Glu Arg Phe Pro Met Met Ser Thr Phe Lys Val Leu Leu Cys Gly Ala Val  
 190 195 200

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FIG. 8C

TTA TCC CGT GTT GAC GCC GGG CAA GAG CAA CTC GGT CGC CGC ATA CAC TAT 663  
 Leu Ser Arg Val Asp Ala Gly Gln Glu Gln Leu Gly Arg Arg Ile His Tyr 220  
 205 210

TCT CAG AAT GAC TTG GTT GAG TAC TCA CCA GTC ACA GAA AAG CAT CTT ACG 714  
 Ser Gln Asn Asp Leu Val Glu Tyr Ser Pro Val Thr Glu Lys His Leu Thr 235  
 225 230

GAT GGC ATG ACA GTA AGA GAA TTA TGC AGT GCT GCC ATA ACC ATG AGT GAT 765  
 Asp Gly Met Thr Val Arg Glu Leu Cys Ser Ala Ala Ile Thr Met Ser Asp 255  
 240 245

AAC ACT GCG GCC AAC TTA CTT CTG ACA ACG ATC GGA GGA CCG AAG GAG CTA 816  
 Asn Thr Ala Ala Asn Leu Leu Leu Thr Thr Ile Gly Gly Pro Lys Glu Leu 270  
 260 265

ACC GCT TTT TTG CAC AAC ATG GGG GAT CAT GTA ACT CGC CTT GAT CGT TGG 867  
 Thr Ala Phe Leu Leu His Asn Met Gly Asp His Val Thr Arg Leu Asp Arg Trp 285  
 275 280

GAA CCG GAG CTG AAT GAA GCC ATA CCA AAC GAC GAG CGT GAC ACC ACG ATG 918  
 Glu Pro Glu Leu Asn Glu Ala Ile Pro Asn Asp Glu Arg Asp Thr Thr Met 305  
 290 295

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FIG. 8D

CCT GCA GCA ATG GCA ACA ACG TTG CGC AAA CTA TTA ACT GGC GAA CTA CTT 969  
 Pro Ala Ala Met Ala Thr Thr Leu Arg Lys Leu Thr Gly Glu Leu Leu 320  
 310  
 ACT CTA GCT TCC CGG CAA CAA TTA ATA GAC TGG ATG GAG GCG GAT AAA GTT 1020  
 Thr Leu Ala Ser Arg Gln Gln Leu Ile Asp Trp Met Glu Ala Asp Lys Val 340  
 325 330 335  
 GCA GGA CCA CTT CTG CGC TCG GCC CTT CCG GCT GGC TGG TTT ATT CGT GAT 1071  
 Ala Gly Pro Leu Leu Arg Ser Ala Leu Pro Ala Gly Trp Phe Ile Ala Asp 355  
 345 350  
 AAA TCT GGA GCC GGT GAG CGT GGG TCT CGC GGT ATC ATT GCA GCA CTG GGG 1122  
 Lys Ser Gly Ala Ala Gly Glu Arg Gly Ser Arg Gly Ile Ile Ala Ala Leu Gly 370  
 360 365  
 CCA GAT GGT AAG CCC TCC CGT ATC GTA GTT ATC TAC ACG ACG GGG AGT CAG 1173  
 Pro Asp Gly Lys Pro Ser Arg Ile Val Val Ile Tyr Thr Thr Gly Ser Gln 390  
 375 380 385  
 GCA ACT ATG GAT GAA CGA AAT AGA CAG ATC GCT GAG ATA GGT GCC TCA CTG 1224  
 Ala Thr Met Asp Glu Arg Arg Asn Arg Gln Ile Ala Glu Ile Gly Ala Ser Leu 405  
 395 400  
 ATT AAG CAT TGG TAACTGTCAG ACCAAGTTTA CTCATATATA CTTTAGA 1273  
 Ile Lys His Trp 410 412

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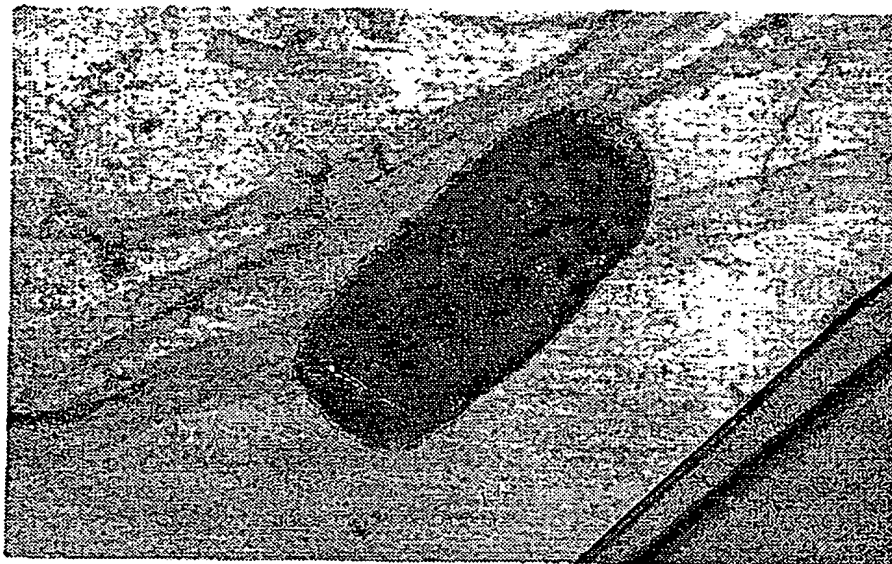


FIG. 9

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US92/09756**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(5) : C12N 1/21, 15/00; A61K 37/02, 39/395

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/27; 530/324, 350, 387.1, 399; 435/7.2, 7.32, 7.35, 7.37, 69.7, 69.8, 172.1, 252.1, 252.3, 252.33, 320.1; 514/2, 12

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS and DIALOG (files 5, 155, 351, 357, 358) search terms: Escherichia, coli, surface, outer membrane, gram negative, anchor, surface display

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
<u>X</u> Y	APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Volume 55, Number 4, issued April 1989, G. Thiry et al., "Cloning of DNA Sequences Encoding Foreign Peptides and Their Expression in the K88 Pili", pages 984-993, see entire document.	1,7,9,13- <u>14, 17</u> 2-3,10,15-16
<u>X</u> Y	JOURNAL OF BACTERIOLOGY, Volume 171, Number 11, issued November 1989, C.K. Murphy et al., "Export of FepA::PhoA Fusion Proteins to the Outer Membrane of <u>Escherichia coli</u> K-12", pages 5894-5900, see entire document.	1-3, 7, 9,11,13, <u>14,17</u> 15-16
<u>X</u> Y	MOLECULAR MICROBIOLOGY, Volume 4, Number 7, issued 1990, M.G. Komacker et al., "The normally periplasmic enzyme $\beta$ -lactamase is specifically and efficiently translocated through the <u>Escherichia coli</u> outer membrane when it is fused to the cell-surface enzyme pullulanase", pages 1101-1109, see entire document.	1,9,11,13- <u>14,17</u> 2-3,7,15-16



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A document defining the general state of the art which is not considered to be part of particular relevance	*X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E earlier document published on or after the international filing date	*Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z document member of the same patent family
*O document referring to an oral disclosure, use, exhibition or other means	
*P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

23 December 1992

Date of mailing of the international search report

JAN 1993

Name and mailing address of the ISA/  
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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US92/09756

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim N .
<u>X</u> Y	THE EMBO JOURNAL, Volume 5, Number 11, issued 1986, A. Charbit et al., "Probing the topology of a bacterial membrane protein by genetic insertion of a bacterial membrane protein by genetic insertion of a foreign epitope; expression at the cell surface", pages 3029-3037, see entire document.	<u>1,5,7,9,13-14,17</u> 2-3,15-16,23-25
<u>X</u> Y	GENE, Volume 59, issued 1987, M. Agterberg et al., "Use of outer membrane protein PhoE as a carrier for the transport of a foreign antigenic determinant to the cell surface of <i>Escherichia coli</i> K-12", pages 145-150, see entire document.	<u>1,5,7-9,13-14</u> 2-3,15-16,23-25
<u>X</u> Y	RES. MICROBIOL., Volume 141, issued 1990, S. Pistor et al., "OmpA-Haemagglutinin Fusion Proteins for Oral Immunization with Live Attenuated <i>Salmonella</i> ", pages 879-881, see entire document.	<u>1,5,8-9,13-14,17,23-25</u> 2-3,10,15-16
<u>X</u> Y	THE EMBO JOURNAL, Volume 9, Number 6, issued 1990, T. Klauser et al., "Extracellular transport of cholera toxin B subunit using <i>Neisseria</i> IgA protease $\beta$ -domain: conformation-dependent outer membrane translocation", pages 1991-1999, see entire document.	<u>1,9,12,14,17</u> 15-16
<u>X</u> Y	GENE, volume 83, issued 1989, F.A. Jacobs et al., "Human metallothionein-II is synthesized as a stable membrane-localized fusion protein in <i>Escherichia coli</i> ", pages 95-103, see entire document.	<u>1-4,9,13-14,17</u> 6,15-16,20-22
<u>X,P</u> Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, Volume 87, Number 7, issued 01 April 1992, J.A. Francisco et al., "Transport and anchoring of $\beta$ -lactamase to the external surface of <i>Escherichia coli</i> ", pages 2713-2717, see entire document.	<u>1-9,11,13-14,16-19</u> 10,12,15,20-25
Y,P	ABSTR. PAP. AM. CHEM. SOC., Volume 203, Part 1, Abstract BIOT 116, issued 1992, J.A. Francisco et al., "Developments and applications of expression systems for the anchoring of proteins on the surface of Gram-negative bacteria", see entire abstract.	1-25

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US92/09756

A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

536/27; 530/324, 350, 387.1, 399; 435/7.2, 7.32, 7.35, 7.37, 69.7, 69.8, 172.1, 252.1, 252.3, 252.33, 320.1; 514/2,  
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